

"CYTOCHROME P450 REDUCTASES FROM POPPY PLANTS"

TECHNICAL FIELD

The present invention relates to production of alkaloids from poppy plants and in particular to genes encoding enzymes in the alkaloid pathway, to proteins encoded by the genes, to plants transformed or transfected with the genes and to methods of altering alkaloid content or blend of poppy plants.

INTRODUCTION

The opium poppy *Papaver somniferum* is grown under strict government control, for the production of medically useful alkaloids such as morphine and codeine. The alkaloid content of poppy straw (includes threshed poppy capsules) is the most important parameter in the efficiency of opium alkaloid production. There have been numerous attempts to increase the yield of alkaloid per ton of poppy material. The vast majority of approaches focus on improving agricultural practices and on established methods of conventional breeding in the attempt to increase cultivation efficiencies and to modifying the genotype of the opium poppy plants.

In addition to increasing the overall yield of opium alkaloids, the relative content of particular alkaloids in the poppy plants is also of considerable importance and has an impact on efficiency of processing of the plant material and the ultimate yield and cost of an alkaloid.

Usually only one of the many alkaloids that can be produced by a poppy plant is found as the predominant alkaloid. In the opium poppy this is predominantly morphine which accumulates after flowering of the plant. However, before flowering thebaine is most abundant. The reason for such bias can be explained, at least in part, by analysis of what is currently known about alkaloid metabolism in the opium poppy and its regulation.

The network of reactions, enzymes, co-factors and metabolic intermediates leading to the synthesis of alkaloids in the opium poppy constitute a complex metabolic pathway which is regulated at numerous points. There are also thought to be a number of rate limiting steps ("bottlenecks") where limitations in the availability of either substrates, co-factors or certain enzymes, determine which particular branch of the synthetic pathway is favoured and therefore the ultimate "mix" of alkaloids and the type of alkaloid which is predominantly in the plant. A class of enzymes known as cytochrome P-450 are known to be involved in the synthesis of several intermediates in the pathway. However, unlike the enzymology of mammalian cytochrome P450

enzymes, similar plant enzymes are considerably less abundant (Biochimie 1987, 69:743-752) and have been less clearly described. It is known that plant P450 enzymes are like mammalian proteins and that they are hemoproteins which have a common prosthetic group containing iron and are membrane-bound proteins found within the endoplasmic reticulum. Generally, the P450-dependent enzymes catalyse the transferral of oxygen to the substrate and effectively remove one of the atoms from an oxygen molecule and are also referred to as monooxygenases. The reactions are dependent on a range of co-factors including NADPH and a second enzyme P450 cytochrome reductase.

More particularly, in the biosynthesis of alkaloids in plants, cytochrome P-450-dependent oxidases and monooxygenases have been shown to catalyse highly regio- and stereoselective reactions. Hydroxylases and oxidases specific to alkaloid biosynthesis have been identified and characterised for the protopine, berberine, bisbenzylisoquinoline, benzophenanthridine, morphinan and monoterpenoid indole alkaloid biosynthetic pathways. The role of cytochrome P-450s in alkaloid biosynthesis is exemplified by the biosynthesis of sanguinarine in *Eschscholzia californica* (California poppy). Of the six oxidative transformations involved in the conversion of (*S*)-reticuline to sanguinarine, four are thought to be catalysed by cytochrome P-450-dependent enzymes.

Thus, plant cytochrome P-450-dependent enzymes, including those from the alkaloid poppy, constitute a substrate-specific class of enzymes that differs from their mammalian counterpart in the high regio- and stereospecificity as well as in the novel nature of the reactions catalysed. The enzymes involved in the alkaloid biosynthetic pathway require among other things the presence of a cytochrome P-450 reductase enzyme. Plant cytochrome P-450 reductases have been purified or enriched from *C. roseus* (1, 8) sweet potato (9), *Helianthus tuberosus* (Jerusalem artichoke) (10), *Glycine max* (soybean) cell suspension cultures (11), *Pueraria lobata* (12) and petunia flowers (13). cDNA encoding cytochrome P-450 reductase has been isolated from *Vigna radiata* (mung bean) (14), *C. roseus* (15), *H. tuberosus* (accession Z26250, Z26251), *Vicia sativa* (accession Z26252) and *Arabidopsis* (16). cDNA cloning and heterologous expression in *E. coli* of the *C. roseus* cytochrome P-450 reductase has been reported (15).

The reductase is responsible for providing electrons to the P450 and is thought to be a relatively promiscuous enzyme in that a particular reductase species will reduce a range of distinct P450s. It is also known that the cytochrome P450 enzymes are in molar

excess to the level of P450 reductase. This imbalance may be a regulatory step for the reduction and therefore be rate-limiting of the cytochrome P450 activity. Although there is some promiscuity within species, available data suggests that there is poor transferability of reductases from diverged species. For example, although cytochrome P-450 reductase from insect cell culture and porcine liver was shown to transfer electrons to heterologously expressed *Berberis* berbaminine synthase, the highest turnover number was achieved with the *Berberis* reductase (7).

Notwithstanding this body of work, to date it has not been possible to establish the exact nature of the "bottlenecks" in the alkaloid metabolism pathway or to identify the key enzymes which may be responsible and which could be used to manipulate alkaloid metabolism in the opium poppy in order to achieve higher yields of alkaloids generally, and specific alkaloids in particular.

As the cost of producing poppy alkaloids is very dependent on the alkaloid content of poppy straw, it would be a major advantage if high alkaloid containing straw could be obtained rather than to attempt to increase the yield of straw. In fact, it is possible that any increase in the yield of straw may result in the relative content of alkaloid decreasing through dilution. High alkaloid-containing straw would provide efficiencies throughout the CPS ("Concentrate of Poppy Straw") production process. If high crop yields can be achieved, either less hectares of crop need be grown or the pre-existing areas can be used to increase production. High yield crops would also reduce the cost of harvest, transport, drying, storage, processing and waste disposal per unit weight of alkaloid. Thus, to increase the yield of an alkaloid it would be most efficient to manipulate the plants to increase alkaloid content of the straw rather than to increase the yield of straw.

SUMMARY OF THE INVENTION

It has now been found that among the rate-limiting steps in the production of morphine in *Papaver somniferum* are the steps which depend on the cytochrome P-450 enzymes, and therefore in turn on the cytochrome P-450 reductases. This observation has led to the identification and isolation of cytochrome P-450 reductase enzymes in the alkaloid poppy, the isolation and characterisation of polynucleotides encoding the reductase enzymes, the expression of the polynucleotides encoding the reductases in eukaryotic and prokaryotic expression systems, including plant cells and transfected or transformed plants. The identification of cytochrome P-450 reductase genes and their products in poppy plants now enables methods of controlling the total alkaloid content

of a plant, the ultimate "mix" of alkaloids as well as the type of predominant alkaloid synthesised by the plant. This can be achieved by alleviating the "bottlenecks" in the pathway through overexpression of the relevant reductase genes in plants transformed or transfected with a nucleotide sequence encoding an appropriate P-450 reductase enzyme.

5 Thus, according to a first aspect there is an isolated and purified polynucleotide encoding a cytochrome P-450 reductase enzyme from an alkaloid poppy plant, or a variant, fragment or analog thereof.

 The polynucleotide may be selected from the group consisting of genomic DNA (gDNA), cDNA, or synthetic DNA. The preferred polynucleotides encoding a
10 cytochrome P-450 reductase are selected from those shown in Figures 9a and 9b or fragments thereof. It will be understood however that sequences shown in the Figures 9a and 9b may be expressed in the absence of the native leader sequences or any of the 5' or 3' untranslated regions of the polynucleotide. Such regions of the polynucleotide may be replaced with exogenous control/regulatory sequences in order to optimise/enhance
15 expression of the sequence in an expression system. Figures 10a and 10b represent examples of truncated polynucleotide sequences encoding cytochrome P-450 reductases of *P. somniferum* and *E. californica* respectively, wherein the native leader sequences have been removed in order to enhance the expression of the enzyme.

 The preferred alkaloid-producing poppy plants are *Eschscholzia californica* and
20 *Papaver somniferum*.

 It will also be understood that analogues and variants of the polynucleotide encoding a cytochrome P-450 reductase from alkaloid poppy plants fall within the scope of the present invention. Such variants will still encode an enzyme with cytochrome P-450 reductase properties and may include codon substitutions or modifications which do
25 not alter the amino acid encoded by the codon but which enable efficient expression of the polynucleotide encoding cytochrome P-450 reductase enzyme in a chosen expression system. Other variants may be naturally occurring, for example allelic variants or isoforms.

 According to a second aspect there is provided an isolated and purified
30 polynucleotide having a sequence which is complementary to all or part of the sequence of a polynucleotide according to the first aspect.

 Such complementary polynucleotides are useful in the present invention as probes and primers, as antisense agents or may be used in the design of other suppressive agents such as ribozymes and the like.

According to a third aspect there is provided an isolated and purified polynucleotide which codes for prokaryotic or eukaryotic expression of a cytochrome P-450 reductase enzyme from an alkaloid poppy plant, or a variant, analog or fragment thereof, wherein the polynucleotide is expressed in an environment selected from the group consisting of the extracellular environment, an intracellular membranous compartment, intracellular cytoplasmic compartment or combinations thereof.

The polynucleotide encoding a cytochrome P-450 reductase may be coupled to another nucleotide sequence which would assist or directing the expression of the reductase with respect to a particular cellular compartment or the extracellular environment.

According to a fourth aspect there is provided a recombinant DNA construct comprising the polynucleotide according to any one of first to third aspects.

Preferably the recombinant DNA construct is a viral or plasmid vector. Such a vector may direct prokaryotic or eukaryotic expression of the polynucleotide encoding a cytochrome P-450 reductase or it may prevent or reduce its expression.

According to a fifth aspect there is provided an isolated and purified cytochrome P-450 reductase enzyme, being a product of prokaryotic or eukaryotic expression of the polynucleotide of any one of first to third aspects or a DNA construct of the fourth aspect.

Variants of the cytochrome P-450 reductase enzyme which incorporate amino acid deletions, substitutions, additions or combinations thereof, are also contemplated. The variants can be advantageously prepared by introducing appropriate codon mutations, deletions, insertions or combinations thereof, into the polynucleotide encoding the P-450 reductase enzyme. Such variants will retain the properties of the P-450 reductase enzyme, either *in vivo* or *in vitro*. Other variants may be naturally occurring, for example allelic variants or isoforms.

The cytochrome P-450 reductase may be expressed in and by a variety of eukaryotic and prokaryotic cells and organisms., including bacteria, yeasts, insect cells, mammalian and other vertebrate cells, or plant cells. Preferably the expression system is a plant expression system and even more preferred is an alkaloid poppy plant. Suitable alkaloid poppy plants are *Eschscholzia californica* and *Papaver somniferum*.

For expression of cytochrome P-450 reductase activity, a fragment of the polynucleotide encoding a cytochrome P-450 reductase may be employed, such fragment encodes functionally relevant regions, motifs or domains of the reductase

protein. Similarly, fragments of the P-450 reductase enzyme resulting from the recombinant expression of the polynucleotide may be used. Functionally important domains of cytochrome P-450 reductase may be represented by individual exons or may be identified as being highly conserved regions of the protein molecule. Those parts of the cytochrome P-450 reductase which are not highly conserved may have important functional properties in a particular expression system.

According to a sixth aspect there is provided a cell transformed or transfected with a polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect.

Cells which may be transfected or transformed with a polynucleotide encoding a cytochrome P-450 reductase are bacterial, yeast, animal or plant cells. For preference the cells are plant cells. Even more preferred are cells from an alkaloid poppy plant, such as *Eschscholzia californica* or *Papaver somniferum*.

According to a seventh aspect there is provided a method for preparing plants which overexpress a cytochrome P-450 reductase enzyme, comprising transfecting or transforming a plant cell, a plant part or a plant, with the polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect.

Preferably, the plants overexpressing the P-450 reductase are *Eschscholzia californica* and *Papaver somniferum*. Suitable promoters to control the expression of the P-450 reductase gene may be derived from for example cauliflower mosaic virus or subterranean clover mosaic virus. Other virus promoters may also be suitable. Further, the use of the endogenous promoter may also be appropriate in certain circumstances. Such a promoter may be co-isolated with the gDNA encoding the P-450 reductase enzyme.

According to a eighth aspect there is provided a plant transformed or transfected with a polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect, wherein the plant exhibits altered expression of the cytochrome P-450 reductase enzyme

For preference, the altered expression manifests itself in overexpression of the cytochrome P-450 reductase enzyme. However, reduced expression of cytochrome P-450 reductase can also be achieved if the plant is transformed or transfected with a polynucleotide which is complementary to the polynucleotide encoding the reductase.

Even more preferably, the transformed or transfected plant is an alkaloid poppy plant, wherein the plant has a higher or different alkaloid content when compared to a plant which has not been so transformed or transfected.

5 Preferably the transformed or transfected plants having higher or different alkaloid content are selected from *Eschscholzia californica* and *Papaver somniferum*. Even more preferred is *Papaver somniferum*.

According to a ninth aspect there is provided a method of altering the yield or type of alkaloid in a plant comprising transforming or transfecting a plant cell, a plant part or a plant with a polynucleotide encoding a cytochrome P-450 reductase enzyme or
10 a variant, analog or fragment thereof, or with a polynucleotide which binds under stringent conditions to the polynucleotide encoding said enzyme.

According to a tenth aspect there is provided a method of increasing the yield of alkaloid in a plant comprising transforming or transfecting a plant cell, a plant part or a plant with a polynucleotide encoding a cytochrome P-450 reductase enzyme or a variant,
15 analog or fragment thereof, wherein the enzyme is overexpressed in said plant

According to a eleventh aspect there is provided a method of altering type or blend of alkaloid in a plant comprising transforming or transfecting a plant cell, a plant part or a plant with a polynucleotide encoding a cytochrome P-450 reductase enzyme or a variant, analog or fragment thereof, or with a polynucleotide which binds under
20 stringent conditions to the polynucleotide encoding said enzyme.

According to a twelfth aspect there is provided a stand of stably reproducing alkaloid poppies transformed or transfected with a polynucleotide according to any one first to third aspects or a DNA construct according to the fourth aspect, having altered expression of the cytochrome P-450 reductase enzyme.

25 According to a thirteenth aspect there is provided a stand of stably reproducing alkaloid poppies transformed or transfected with a polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect, having a higher or different alkaloid content when compared to a plant which has not been so transformed or transfected.

30 Preferably the stably reproducing alkaloid poppy is *Papaver somniferum*.

According to a fourteenth aspect there is provided straw of stably reproducing poppies according twelfth or thirteenth aspect, having a higher or different alkaloid content when compared to the straw obtained from an alkaloid poppy which has not been transformed or transfected.

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According to a fifteenth aspect there is provided a concentrate of straw according to the fourteenth aspect, having a higher or different alkaloid content when compared to the concentrate of straw obtained from an alkaloid poppy which has not been transformed or transfected.

5 According to a sixteenth aspect there is provided an alkaloid when isolated from the straw according to fourteenth aspect or the concentrate according to the fifteenth aspect.

According to a seventeenth aspect there is provided a method for the production of poppy plant alkaloids, comprising the steps of;

10 a) harvesting capsules of an alkaloid poppy plant transformed or transfected with a polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect, to produce a straw where the poppy plant is such a plant that the straw has a higher or different alkaloid content when compared to the straw obtained from a poppy plant which has not been transformed or transfected.

15 b) chemically extracting the alkaloids from the straw.

According to an eighteenth aspect there is provided a method for the production of poppy alkaloids, comprising the steps of;

20 a) collecting and drying the latex of the immature capsules of an alkaloid poppy plant transformed or transfected with a polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect, to produce opium where the poppy plant is such a plant that the opium has a higher or different alkaloid content when compared to the opium obtained from a poppy plant which has not been transformed or transfected.

25 b) chemically extracting the alkaloids from the opium.

For preference the alkaloid is morphine, codeine, oripavine or thebaine, but it will be understood that other intermediates in the alkaloid metabolic pathway are also within the scope of the present invention, as are mixtures of alkaloids.

30 Unless the context clearly requires otherwise, throughout the description and the claims, the words 'comprise', 'comprising', and the like are to be construed in an inclusive as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

BRIEF DESCRIPTION OF FIGURES

Figure 1. SDS-PAGE analysis of fractions from the purification of cytochrome P-450 reductase from *P. somniferum* cell suspension cultures. Protein bands were visualised by silver staining. Lane 1, protein standards; lane 2, affinity chromatography elution buffer without protein; lane 3, 1 µg protein from the 2',5'-ADP Sepharose 4B eluate after dialysis; lane 4, 4 µg microsomal protein; lanes 5,6, 4 µg solubilized microsomal protein; lane 7, 4 µg protein from the DEAE cellulose eluate.

Figure 2. Amino acid sequences of seven endoproteinase Lys-C-generated peptides of the cytochrome P-450 reductase from *P. somniferum* cell suspension cultures.

Figure 3. Partial amino acid sequence comparison of plant cytochrome P-450 reductases. The shaded areas and arrows indicate the position and direction of the regions used for PCR oligodeoxynucleotide primer design.

Figure 4. Genomic DNA gel blot analysis of (A) *P. somniferum* hybridized to the *P. somniferum* full-length cDNA and (B) *E. californica* hybridized to the *E. californica* full-length cDNA and to (C) the 288 bp PCR fragment corresponding to the second isoform. The numbers following the restriction enzyme names indicate the number of recognition sites that occur in the reading frame. For the second *E. californica* isoform, this is known only over a 288 bp region.

Figure 5. Comparison of the amino acid sequences of the cytochrome P-450 reductase from *P. somniferum* and from *E. californica*. Top sequence, *E. californica*; bottom sequence, *P. somniferum*; *, amino acid identity.

Figure 6. Nucleotide sequences of cDNA from (a) *P. somniferum*, and (b) *E. californica*.

Figure 7. Functional expression of cytochrome P-450 reductases in yeast and insect cell culture. (A) Expression of pYES2/PsoCPRI (———), pRS405/PsoCPRII (- - - - -), pYES2/PsoCP (———), control (·······); (B) pFastBac/PsoCPRII (———), control (·······); (C) pYES2/EcaCPRII (———), pRS405/EcaCPRII (- - - - -), control (·······); (D) pFastBac/EcaCPRII (———), control (·······). Pso CPR, *P. somniferum* cytochrome P-450 reductase; Eca CPR, *E. californica* cytochrome P-450 reductase; Sf9, *S. frugiperda* Sf9 cell culture.

Figure 8. Restriction enzyme map (unique sites) for cDNA sequences of (a) *P. somniferum*, and (b) *E. californica*.

Figure 9. Amino acid sequences of (a) *P. somniferum*, and (b) *E. californica*, predicted from their respective cDNA nucleotide sequences. The start and stop codons are depicted in bold.

5 **Figure 10.** cDNA nucleotide sequences and their predicted amino acid sequences, of fragments subcloned into an expression vector: (a) *P. somniferum*, and (b) *E. californica*. Both sequences are truncated versions of sequences represented in Figures 9a and 9b, lacking the leader sequences. Extra vector sequences/restriction sites derived during subcloning are shown in lowercase and the cDNA in uppercase.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

10 The details of the metabolic pathway leading to synthesis of opium alkaloids in the opium poppy, *Papaver somniferum*, part of which is depicted in Scheme I. Typically, the P450 enzyme exists in a 15 - 20 fold excess as compared to the reductase level and as there is approximately a 6:1 dependence between the two enzymes, it is feasible that the reductase levels are limiting the rate of the cytochrome P450 enzyme. By supplying plant tissue with radiolabeled compounds and following the accumulation of radioactivity in the various intermediates in the pathway it was shown that addition of radiolabel (labelled reticuline, salutaridinol) before thebaine results in accumulation of radioactivity at thebaine. Addition of radiolabeled compounds after thebaine result in the accumulation of radioactivity at codeine.

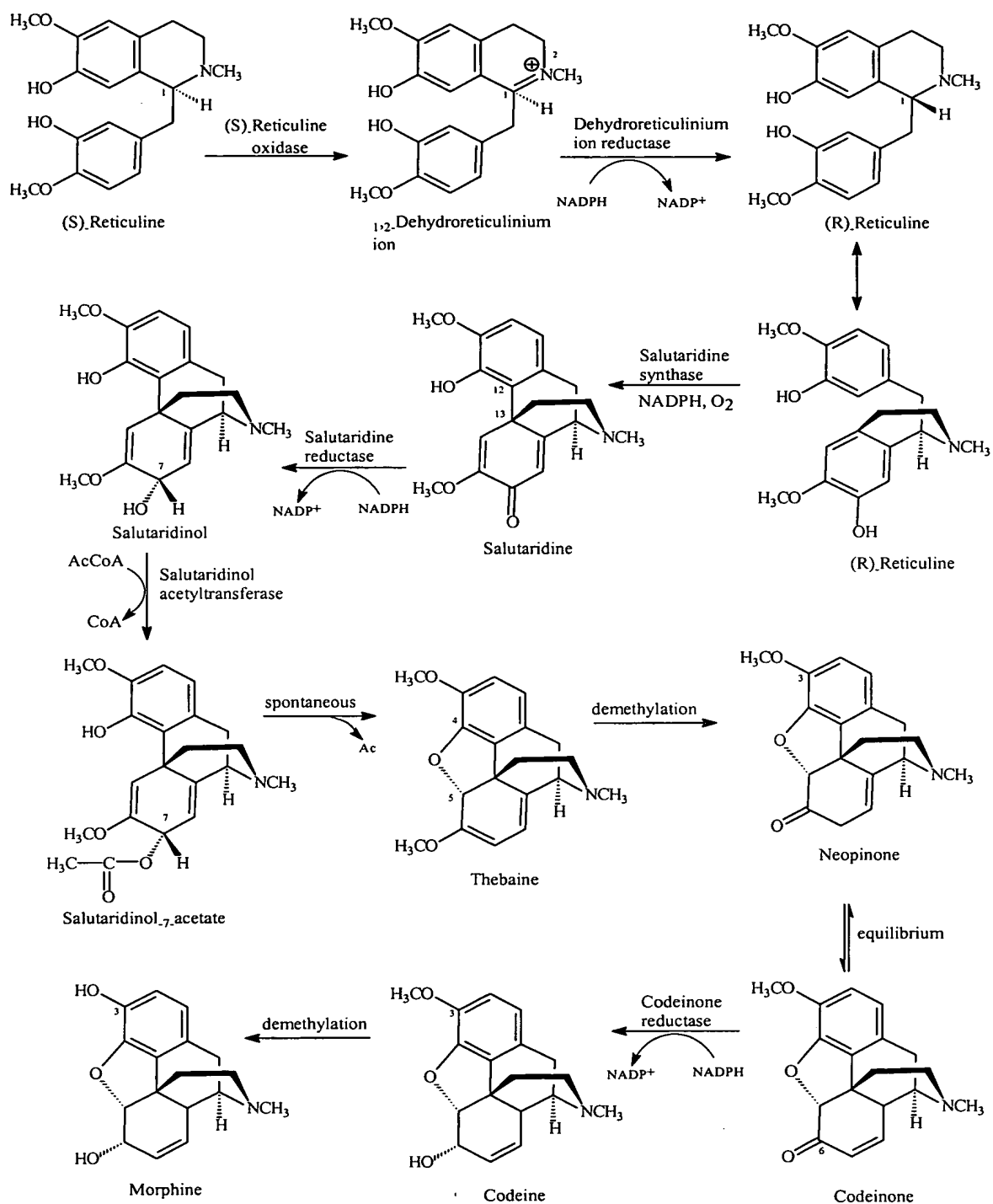
20 Oripavine is an intermediate from a second route of conversion from thebaine to morphine. It is thought that thebaine is converted to oripavine by the same 3 demethylase that converts codeine to morphinone. The slow modification of the isotopic oripavine is probably due to the rate limitation of the 6 demethylase.

With the assistance of such experiments it has now been found that among the rate-limiting steps in the production of morphine in *Papaver somniferum* are the steps which depend on the reduction of cytochrome P-450 by the cytochrome P-450 reductases.

25 Thus the following steps are known or suspected to be catalysed by P-450 enzymes which are rate limiting:

- 1 (R)-reticuline → salutaridine
- 2 thebaine → neopinone → codeinone
- 3 codeine → morphine

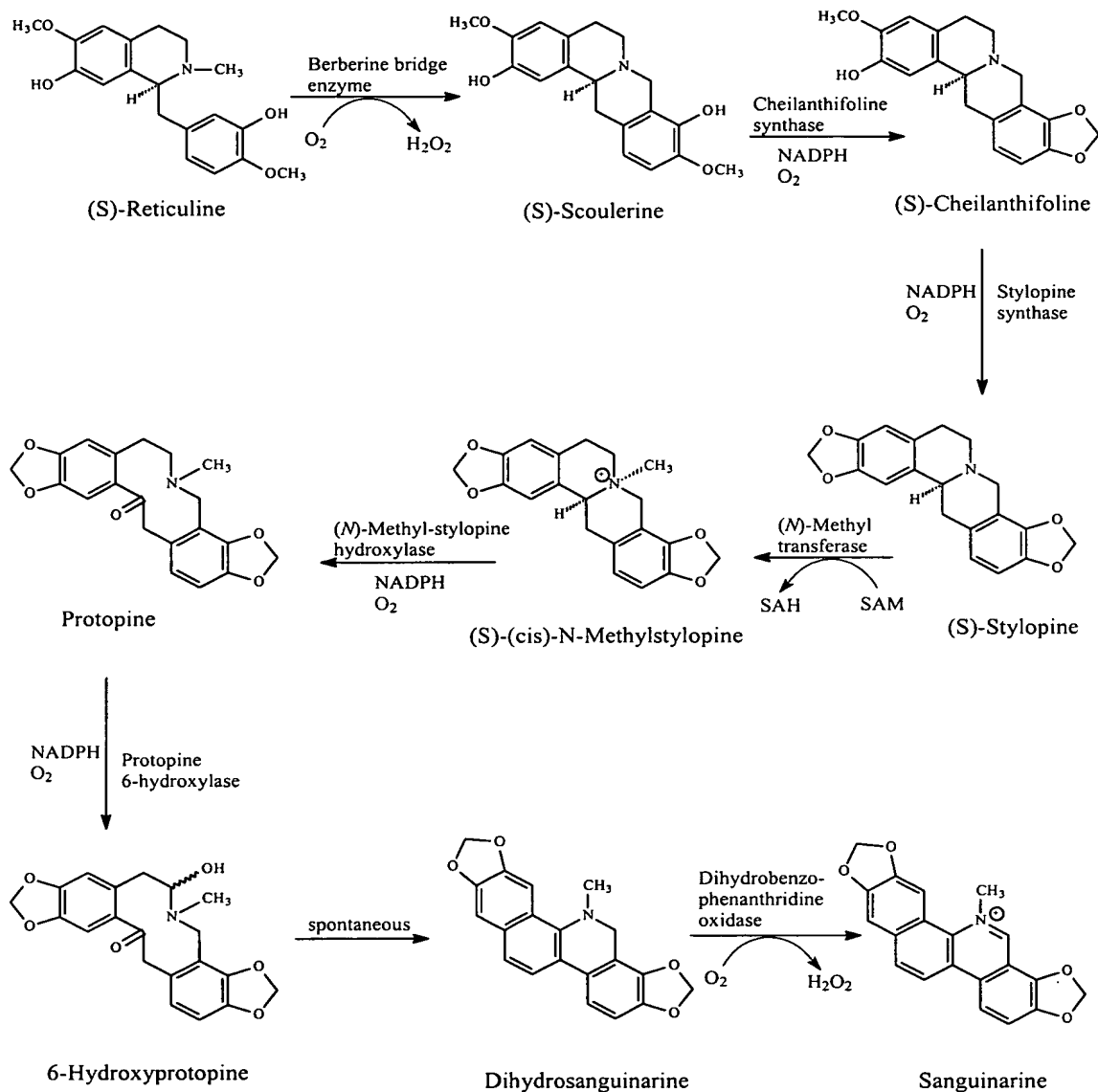
SCHEME I



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The importance of cytochrome P-450 in alkaloid biosynthesis is also exemplified by the biosynthesis of sanguinarine in *Eschscholzia californica* (California poppy). This biosynthetic pathway is shown in Scheme II.

SCHEME II



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The present invention provides by way of example the sequence of the P-450 reductase enzyme genes from two poppy species, the opium poppy *Papaver somniferum* and the Californian poppy *Eschscholzia californica*. The sequence information has been shown to code for the enzymes by expression in a heterologous expression system followed by biochemical characterisation. These studies have also shown that the

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alteration of the ratio and species of reductase will significantly alter the interaction of the cytochrome P-450 with its substrate suggesting a lack of tolerance for general interchange of reductase genes.

5 The over-expression of the P-450 reductase gene in an alkaloid producing plant will alleviate the rate-limitation of the P450 by increasing the rate of reduction of the active P-450 enzyme. In brief, controlling the reductase should control the P-450 cytochrome.

The information on the protein coding region of cytochrome P-450 reductase enzymes may be applied to increase yields of alkaloids in the poppy plant as follows:

- 10 1) obtain the gDNA or cDNA sequence of the gene from the target plant and a closely related plant.
- 2) sub-clone the gDNA or cDNA into a plasmid vector that contains the following:
 - a promoter suitable for overexpression of the cDNA in poppy, for example a promoter derived from the cauliflower mosaic virus or the subterranean clover
 - 15 mosaic virus.
 - a selectable marker linked to a different promoter to facilitate the selection of transformants. Marker could be a dominant marker such as a herbicide resistance gene or an antibiotic resistance gene.
 - suitable selectable markers and replication origins for maintenance of the
 - 20 plasmid in bacteria
 - suitable sequences to facilitate mobilisation of the plasmid by *Agrobacterium tumefaciens*-mediated transformation.
- 3) transform a suitable strain of *A. tumefaciens* and then co-cultivate the bacteria with suitable samples of plant tissue such as callus, embryonic tissue or hypocotyl tissue.
- 25 4) place treated tissue on selectable media and provide appropriate media to promote differentiation and plant re-generation.
- 5) characterise candidate plants by Southern and Northern blotting to confirm integration of gene and expression in appropriate tissues
- 6) self-pollinate transformed plants, analyse segregants to identify hemizygotes and
- 30 homozygotes
- 7) analyse biochemistry of transgenic plants.

Isotope labelling can be used to identify bottlenecks and HPLC analysis will determine levels of alkaloids.

In order to develop optimised convenient heterologous expression systems for the cytochrome P-450-dependent oxidases of select isoquinoline alkaloid-producing plant species, facile PCR-based method have been developed with which to clone cytochrome P-450 reductase and express the enzyme in yeast and insect cell culture as initial expression systems.

The invention will now be described with reference to specific examples.

EXAMPLES

Example 1: Enzyme purification and amino acid sequencing:

Plant cell cultures. Cell suspension cultures of *P. somniferum* and *E. californica* were routinely grown in 1-litre conical flasks containing 400 mL of Linsmaier-Skoog medium (17) over 7 days at 23°C on a gyratory shaker (100 rpm) in diffuse light (750 lux). Elicitation of *E. californica* cell suspension cultures was achieved by the aseptic addition of methyl jasmonate to a final concentration of 100 µM to the medium (18).

Purification and sequence analysis. Cells were harvested from seven-day-old suspension cultures of *P. somniferum* by vacuum filtration, immediately shock frozen and stored at -20°C. All of the following operations were carried out at 4°C. 500 g frozen tissue were then homogenised with a mortar and pestle in 1 litre 0.1 M tricine/NaOH, pH 7.5 containing 15 mM thioglycolic acid. Cell debris was removed by centrifugation at 10,000 x g, 30 min. The supernatant was filtered through four layers of cheesecloth and the microsomes were then isolated by MgCl₂ precipitation according to (19). In a typical preparation, 500 g fresh weight of cells yielded 8-10 mg/mL microsomal protein. Microsomal protein was solubilized as follows. 2 mg CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane-sulfonate, Roth) per mg microsomal protein was prepared in 1 mL of 0.1 M tricine/NaOH, pH 7.5 containing 15 mM thioglycolic acid. This solution was added dropwise to the microsomal suspension. 2% (v/v) Emulgen 911 (Kao Corporation) was then added and the solution slowly stirred for 1 h. Membrane fragments were removed by centrifugation at 105,000 x g for 60 min. The total activity in the solubilized microsomes was assigned the value 100%. The solubilized cytochrome P-450 reductase was then purified to electrophoretic homogeneity according to (20). In this manner, 50 µg cytochrome P-450 reductase was purified from 8 kg *P. somniferum* cell suspension culture in 97% yield.

The purified enzyme preparation was subjected to SDS/PAGE to remove traces of Emulgen 911 and CHAPS, and the Coomassie brilliant blue R-250-visualized band

representing the cytochrome P-450 reductase was digested *in situ* with endoproteinase Lys- C as reported in (21). The peptide mixture thereby obtained was resolved by reversed phase HPLC [column, Merck Lichrospher RP18; 5 μ m (4 x 125 mm); solvent system, (A) 0.1% trifluoroacetic acid, (B) 0.1% trifluoroacetic acid / 60% acetonitrile; gradient of 1% per min; flow rate of 1mL/min] with detection at 206 nm. The scheme for the purification of the cytochrome P-450 reductase is given in Table I.

TABLE I Purification of Cytochrome P-450 Reductase from *P. somniferum* Cell Suspension Cultures

Purification Step	Total protein (mg)	Total activity (nkatal)	Specific activity (nkatal/mg)	Purification factor (fold)	Yield (%)
Microsomes	308	59	0.2	-	-
Solubilized microsomes	244	71	0.3	1	100
DEAE Cellulose	21	71	3.4	11	100
2',5'-ADP Sepharose 4B	0.05	47	927	3100	66
Dialysis	0.05	69	1385	4600	97

Following this facile purification procedure (20), 50 μ g of enzyme could be purified to near electrophoretic homogeneity from 8 kg fresh weight of cell suspension culture with minimal loss of activity. Gel electrophoretic analysis of aliquots of the purification steps suggest that there may be two isoforms of the cytochrome P-450 reductase in *P. somniferum* as there were two protein bands present in the 2',5'-ADP Sepharose 4B eluate at 80 kDa (Fig. 1). To further test the possible presence of isoforms, 10 μ g protein from the 2',5'-ADP Sepharose 4B eluate was subjected to native polyacrylamide gel electrophoresis, the two closely migrating protein bands were eluted and both tested positive for cytochrome *c* reduction. These two isozymes could not be chromatographically resolved and were therefore characterised together.

The purified reductase exhibited a pH optimum at 8.0 in 0.5 M Tricine buffer. The optimal molarity range of the Tricine buffer was determined to be 250-500 mM. At 100 mM and at 1 M Tricine, the activity declined to 21% and 77%, respectively. The K_m value for cytochrome *c* was 8.3 μ M and that for the cofactor NADPH was 4.2 μ M. The distribution of the cytochrome P-450 reductase in a 3-month-old *P. somniferum* plant is given in Table II.

was then resolved by agarose gel electrophoresis, the bands of approximately the correct size were isolated and subcloned into pGEM-T (Promega) prior to nucleotide sequence determination.

Resolution of this first PCR experiment by agarose gel electrophoresis revealed a mixture of DNA products in the expected range of 400-450 bp. The bands in this size range were eluted from the gel and used as template for nested PCR with the following primers:

5'-CA ITI CII CCT CCT TTC CC-3' and
T

3'-AAA CGI CGI TAI CGI GGI GCI IGI GTT GG-5'
G C

The result from the nested PCR was a single DNA band with the expected size of 288 bp. The translation of the nucleotide sequence of this PCR product indicated that it was indeed encoding a cytochrome P-450 reductase. This 288 bp PCR-generated partial cDNA was then used as hybridisation probe to screen an amplified *P. somniferum* cell suspension culture cDNA library. In this manner, from a total of 300,000 clones screened, two positive clones were isolated. Of these two positive clones, one was determine to be full-length by a restriction endonuclease analysis. The nucleotide sequence of this full-length cDNA clone was then determined for both strands. The reading frame coded for 684 amino acids corresponding to a relative molecular mass of 77.5 kDa.

An identical PCR-based approach was also carried out with RNA isolated from methyl jasmonate-induced *E. californica* cell suspension cultures (26). Nucleotide sequence determination of the 288 bp DNA fragment indicated that in *E. californica* one cytochrome P-450 reductase form is present. However, screening of 400,000 clones of a primary cDNA library prepared from RNA isolated from methyl jasmonate-induced *E. californica* cell suspension cultures resulted in the isolation of one partial and one full-length clone, both of which encoded a second isoform. The nucleotide sequence of this full-length cDNA clone was then determined for both strands. The reading frame encoded 705 amino acids that corresponded to a relative molecular mass of 78.7 kDa. RNA gel blot analysis indicated that this isoform gene is weakly induced two-fold by treatment of the cell cultures with methyl jasmonate. Genomic DNA gel blot analysis of

each reductase indicates that one gene encodes each isoform in *E. californica* and that one gene also encodes the cloned isoform in *P. somniferum* (Fig. 4).

The overall sequence homology of the cytochrome P-450 reductase from *P. somniferum* and that from *E. californica* is 63% identity at the nucleotide level and 69% identity at the amino acid level (Fig. 5). This compares to an overall sequence identity to other plant cytochrome P-450 reductases of approximately 50% at both the nucleotide and amino acid levels.

Nucleotide sequence determination. The entire nucleotide sequence on both DNA strands of full-length cDNA clones in pBluescript was determined by dideoxy cycle sequencing using internal DNA sequences for the design of deoxyoligonucleotides as sequencing primers. Nucleotide sequences of cDNAs of *P. somniferum* and *E. californica* are given in Figures 6A and 6B, respectively.

Alternative approaches. cDNA can also be prepared by isolating RNA from either plant cell suspension cultures or from different material, according to a method using LiCl precipitation of ribonucleic acid as described in "Current Protocols in Molecular Biology" Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds John Wiley & Sons, Inc. New York (1987). mRNA was then isolated from the total RNA using either an oligo dT cellulose column or oligo dT beads (Oligotex beads, QIAGEN) according to the manufacturers instructions. The cDNA libraries were prepared from mRNA with cDNA and lambda ZAP kits from Stratagen (La Jolla, California, USA), according to the manufacturers instructions.

Example 3: cDNA isolation and heterologous expression of cytochrome P-450 reductase in *Saccharomyces cerevisiae*.

cDNA clones encoding the *Papaver* and *Eschscholzia* cytochrome P-450 reductases were isolated by screening of cDNA libraries prepared in either l-ZAP II or Uni-ZAP XR (Stratagene) using the partial clones generated by PCR as hybridization probe. The clones that yielded positive results through a third screening were converted to pBluescript KS (+) by excision. After determination of the nucleotide sequence on both strands, the full length reading frame, free of the 5'- and 3'-flanking sequences, was generated by PCR using either *Taq* DNA polymerase (Perkin Elmer) and was subcloned into pGEM-T (Promega) or *Pfu* DNA polymerase and was subcloned into pCR-Script SK (+) (Stratagene).

The *P. somniferum* cytochrome P-450 reductase cDNA in pGEM-T, designated pGEM-T/PsoCPR, was digested with the restriction endonucleases *Not* I and *Hin* dIII and the 2096 bp fragment was ligated into *Not* I/*Hin* dIII digested pYES2 (autonomously replicating yeast expression vector from Invitrogen) to produce the expression plasmid pYES2/PsoCPRI. This particular construction had 27 bp of a noncoding region upstream from the AUG start codon. This was reduced to 6 bp by digestion of pYES2/PsoCPRI with *Hin* dIII and *Cla* I. This 55 bp restriction fragment was then replaced by ligation with a synthetic DNA adaptor of a sequence that replaced the reading frame from the internal *Cla* I site through the start codon, which was immediately preceded by a *Hin* dIII recognition sequence. The resulting construct was termed pYES2/PsoCPRII.

The *E. californica* cytochrome P-450 reductase cDNA in pGEM-T, designated pGEM-T/EcaCPR, was digested with the restriction endonucleases *Sal* I and *Not* I and the 2289 bp fragment was ligated into *Sal* I/*Not* I digested pGEM-9Zf (-) (Promega). pGEM-9Zf/EcaCPR was then digested with *Sst* I and *Not* I and the 2292 bp fragment was ligated into *Sst* I/*Not* I digested pYES2 to produce the expression plasmid pYES2/EcaCPRI. The noncoding sequences upstream of the start codon were minimized by digestion with *Sma* I and *Eco* ICRI and the vector recircularized by blunt-end ligation (plasmid termed pYES2/EcaCPRII). These autonomously replicating expression plasmids were then introduced into the *Saccharomyces cerevisiae* strain INVSC1 under uracil selection.

Expression of the vector pYES2/PsoCPRI containing 27 noncoding nucleotides upstream of the start codon resulted in increased enzyme activity that was 2.6-fold greater than the yeast endogenous reductase (Fig. 7A). Shortening of this noncoding sequence to 6 bp in vector pYES2/PsoCPRII resulted in 9-fold greater enzyme activity than in the control yeast strain containing only the vector pYES2. Expression using the integrative yeast vector pRS405 was also investigated for the eventual possible heterologous co-expression of both a plant cytochrome P-450 reductase and a plant oxidase in yeast. Expression of the vector pRS405/PsoCPRII, in which transcription of the *P. somniferum* cytochrome P-450 reductase was also driven by the *GAL1* gene promoter, resulted in 67% of the enzyme activity compared to the autonomously replicating vector pYES2/PsoCPRII.

Expression of pYES2/EcaCPRII and of pRS405/EcaCPRII resulted in a 15-fold and 10-fold increase in activity over the endogenous yeast reductase, respectively (Fig. 7C).

5 The *P. somniferum* cytochrome P-450 reductase reading frame downstream from the GAL 1 promoter was generated by PCR from pYES2/PsoCPRII. The 2598 bp PCR product was ligated into pCRScript and then excised by digestion with *Not* I and *Sal* I. This 2669 bp *Not* I/*Sal* I fragment was ligated into the *Not* I/*Sal* I digested yeast integrative expression vector pRS405 (Stratagene).

10 The *E. californica* cytochrome P-450 reductase reading frame downstream from the GAL 1 promoter was introduced into the yeast integrative expression vector pRS405 by digestion of pYES2/EcaCPRII with *Pst* I and *Not* I and the 2835 bp fragment was ligated into *Pst* I/*Not* I digested vector. The integrative expression plasmids pRS405/PsoCPRII and pRS405/EcaCPRII were then introduced into the *S. cerevisiae* strain INVSC1 under leucine selection.

15 Yeast microsomes were isolated according to either (22) or (23) and the presence cytochrome P-450 reductase was measured as the ability to reduce cytochrome *c* (24).

Example 4: Heterologous expression of cytochrome P-450 reductase in *Spodoptera frugiperda* Sf9 cells.

20 The *P. somniferum* cytochrome P-450 reductase cDNA construct pYES2/PsoCPRII was digested with *Hin* dIII and *Xba* I and the resultant 2096 bp fragment was ligated into *Hin* dIII/*Xba* I digested pGEM-7Zf (+) (Promega). pGEM-7Zf/PsoCPRII was then digested with *Bam* HI and *Xho* I and the 2090 bp fragment was ligated into *Bam* HI/*Xho* I digested pFastBac1 (Life Technologies).

25 The *E. californica* cytochrome P-450 reductase clone pGEM-T/EcaCPRII was digested with the restriction endonucleases *Sma* I and *Not* I and the 2251 bp fragment was ligated into pFastBac1 that had been digested first with *Bam* HI, then with *Pfu* DNA polymerase to produce blunt ends, and finally with *Not* I. pFastBac/PsoCPRII and pFastBac/EcaCPRII were transposed into baculovirus DNA and then transfected into *Spodoptera frugiperda* Sf9 cells according to the manufacturer's instructions. The insect
30 cells were propagated and the recombinant virus was amplified according to (7). Isolation of insect cell microsomes was performed as published (7) and the cytochrome *c* reducing activity measured as for the yeast expression.

Heterologous expression in insect cell culture (*S. frugiperda* Sf9 cells) of pFastBac/PsoCPRII produced 4-fold more activity than the insect cell endogenous reductase, representing 40% of the activity produced by pYES2/PsoCPRII in yeast (Fig. 7B). Expression of pFastBac/PsoCPRI, the construction containing a 27 bp long 5'-noncoding region, resulted in no measurable enzyme activity above that from the endogenous insect cell reductase.

Expression of pFastBac/EcaCPRII in insect cell culture produced a 10-fold increase in reductase activity (Fig 7D). The overexpression in insect cell culture was 54% of that achieved in yeast.

Example 5: Co-expression of cytochrome P-450 reductase and berbamunine synthase in Sf9 cells.

To test for the possible effects of a plant cytochrome P-450 reductase as opposed to either yeast or insect cell reductase, several coexpressions were undertaken.

Recombinant baculovirus containing either the *P. somniferum* or *E. californica* cytochrome P-450 cDNA was added simultaneously to *S. frugiperda* Sf9 cells (Gibco-BRL) with recombinant virus containing the berbamunine synthase (CYP 80) cDNA (7). The oxidase virus was infected at a multiplicity of infection (MOI) of approximately 5 and the amount of reductase virus varied from an MOI from 1-5. The infection were carried out as described in (25).

The cytochrome P-450 oxidase that was used in these experiments was the C-O phenol coupling enzyme of bisbenzylisoquinoline alkaloid biosynthesis from *B. stolonifera*, berbamunine synthase (7). In the presence of equimolar concentrations of the two substrates (*S*)-*N*-methylcoclaurine and (*R*)-*N*-methylcoclaurine, the native enzyme produces two products in a ratio of 90:10 (berbamunine (*R,S*-dimer):guattegaumerine (*R,R*-dimer)) that correspond to the ratio of these two alkaloids found in the *Berberis* plant (6). Berbamunine synthase expressed in insect cells produced, however, the dimers *R,S*:*R,R* in a ratio of 15:85 (7). Co-infection of insect cell culture with two baculovirus preparations, one containing berbamunine synthase cDNA and the other containing *E. californica* cytochrome P-450 reductase, in varying ratios resulted in a shift in the ratio of the enzymatic products formed as follows: oxidase:reductase (5:1), *R,S*:*R,R* (29:71); oxidase:reductase (1:1), *R,S*:*R,R* (35:65); oxidase:reductase (1:2), *R,S*:*R,R* (37:63).

The isolation and functional expression of cDNAs encoding cytochrome P-450 reductases from *E. californica* and *P. somniferum* described above were undertaken to develop suitable heterologous expression systems optimal for the active expression of select cytochrome P-450-dependent oxidases of alkaloid biosynthesis, thus providing a convenient test system. Initial characterisation of the cytochrome P-450 reductase from *P. somniferum* indicated that with respect to molecular weight, K_m and pH optimum, the reductase is similar to those characterised from other plant species (9,13,14). The purified reductase resolved into two closely migrating bands on SDS-PAGE, suggesting that isoforms are present in *P. somniferum*. This is similar to the finding that multiple reductase isoforms are present in *Arabidopsis thaliana* (16) and *H. tuberosus*. The presence of isoforms in *P. somniferum* was further supported by amino acid sequence analysis of the purified reductase as compared to the sequence identified through cDNA cloning. In addition, isolation of a cDNA encoding cytochrome P-450 reductase from *E. californica* indicated the presence of two isoforms in this plant species as well. The presence of at least two genes in each genome was corroborated by genomic DNA gel blot analysis.

The cDNA encoding one cytochrome P-450 reductase isoform from each *P. somniferum* and *E. californica* was functionally expressed in yeast in an autonomously replicating vector and in an integrative vector with transcription under the control of the *GAL1* gene promoter. These vector constructions resulted in a 6- to 15-fold increase in reductase activity as compared to the activity from the endogenous yeast reductase alone. Likewise, expression of the reductases in insect cell culture using a baculovirus expression vector produced a 4- to 10-fold increase in reductase activity. Improved heterologous expression was obtained when the 5'- noncoding sequences were completely removed from the cDNAs.

Co-expression of the *Eschscholzia* reductase with the plant oxidase berbaminine synthase (7) in insect cell culture indicated that the amount of plant reductase present exerted an influence on the ratio of the products that were enzymatically formed. A first indication of this effect was shown by reconstitution of purified heterologously expressed berbaminine synthase reconstituted with *Berberis* reductase or with porcine reductase (7). Since it is difficult to standardise the lipids when reductase and oxidase are purified from microsomal membranes originating from different organisms, a co-infection of insect cells with reductase and oxidase is one method by which to avoid the varying effects of lipids. An increasing amount of plant reductase resulted in a shift in

the ratio of products formed by berbaminine synthase from *R,S:R,R* in a ratio of 15:85 in the absence of *Eschscholzia* reductase to *R,S:R,R* (37:63) when a two-fold excess of baculovirus containing the *Eschscholzia* reductase was used for the co-infection. These results indicate that the cytochrome P-450 reductase may influence the binding of substrate to berbaminine synthase. Although the FMN, FAD and NADPH-binding domains of cytochrome P-450 reductase have been identified by sequence comparisons with well studied flavoproteins, less is known about the substrate binding sites (27,28). Interaction with the non-physiological substrate cytochrome *c* has been demonstrated by chemical cross-linking (29) and by site-directed mutagenesis (30) to involve an acidic region between amino acid residues 200-220 of rat cytochrome P-450 reductase, but an elucidation of the specific interaction between reductase and cytochrome P-450 has not yet been reported.

Example 6: Transformation of poppy plant cells with nucleotide sequences encoding cytochrome P-450 reductase proteins.

15 Plant material

The genotypes of *Papaver somniferum* used was C 048-6-14-64 obtained from Tasmanian Alkaloids, Australia. Seeds were surface sterilised by washing for 30-60 seconds in 70% ethanol then in 1%(w/v) sodium hypochlorite solution plus 1-2 drops of autoclaved Tween 20 or Triton X for 20 minutes with agitation. Seeds were rinsed three to four times in sterile distilled water or until no smell of bleach remains and placed on 90 x 25 mm Petri dishes containing B5O medium (see below). Dishes were sealed with Micropore tape and were usually stored at 4°C for 24 to 48 hours. Seeds were germinated at 24°C in a 16 hour light-8 hour dark cycle. Hypocotyls were excised from seedlings after 7-8 days of culture and were cut into 3-6 mm explants (usually 1-3 explants per seedling) and used in transformation experiments.

25 Tissue culture media and conditions

All culture media consisted of B5 macronutrients, micronutrients, iron salts and vitamins (32) and 20g/L sucrose. pH was adjusted with 1M KOH to pH 5.6, media was buffered with 10mM MES (2-[N-Morpholino]ethanesulfonic acid) and the gelling agent was 0.8% Sigma Agar. Growth regulators were added to media prior to autoclaving at 121°C for 20 minutes. B5O has no growth regulators and Callusing Medium (CM) has 1mg/L 2,4-D. Antibiotics were added after autoclaving and cooling to 55-65°C. Explant and type I callus cultures were grown in Petri dishes sealed with Micropore tape at 24°C. Type II callus and somatic embryos were cultured at 18°C.

Bacterial strains and binary vectors

The disarmed *Agrobacterium tumefaciens* strains AGLO and AGL1 (33) were used in transformation experiments. DNA constructs were based on the binary vector pPZP201 (34), e.g. pTAB101, with 35S 5':pat:35S 3'. *Agrobacterium* strains were
5 maintained in glycerol at -80°C or on LB agar plates plus appropriate selection at 4°C. Fresh cultures were grown overnight at 28°C in 10 mL MG broth (35) without antibiotics. This *Agrobacterium* suspension was diluted to approximately 5×10^8 cells mL^{-1} ($\text{OD}_{600} = 0.25$) for use in transformation experiments.

Transformation and embryogenesis

10 Hypocotyls were excised from seedlings and immediately inoculated by immersion in liquid *Agrobacterium* culture for 10-15 minutes. Explants were then transferred directly to CM. After four to five days co-cultivation explants were washed in sterile distilled water, until the water was clear of *Agrobacterium*, blotted on sterile filter paper and transferred to CM containing 150 mg/L Timentin plus 10 mg/L PPT
15 (phosphinothricin, the active ingredient of Basta herbicide). Explants were transferred to fresh CM at three weekly intervals. They initially produced friable brownish type I callus which subsequently formed small regions of very white, compact embryogenic callus (type II) by about 7-8 weeks culture.

Type II callus was transferred to B5O containing 150 mg/L Timentin plus 10
20 mg/L PPT and cultures were transferred to fresh medium every three weeks. Meristemoid/embryo development usually occurred after one or two periods on B5O medium and were seen from about 14-16 weeks total culture time.

Plantlet development from embryos was slow and required a further 3 months in tissue culture before shoot and root growth was sufficient to ensure successful
25 transplantation to soil.

If the initial pH of the medium was 5.8 and MES was omitted, the pH of poppy cultures rapidly rose to pH 8.0 or higher. Fresh agar-solidified B5-based medium adjusted to pH 5.6 rose to $\text{pH} > 6.4$ in the immediate area around type II callus within 30 mins. The inclusion of chlorophenol red in the medium was used to observe these
30 localised increases in pH; the medium turns purple at $\text{pH} 6.4$. The whole plate was $\text{pH} > 7$ within 24 h. At the end of the culture period pH values were measured at 8.7. This rapid rise in pH resulted in very poor growth which is not compensated for by frequent

changes of medium. The rapid rise was significantly delayed even by 2.5 mM MES, but 10 mM MES was preferred to adequately buffer the medium and support improved growth over the 3 week subculture period.

5 The identification and cloning of genes for cytochrome P-450 reductase enzymes now provides means by which the pathway of alkaloid metabolism can be regulated, specifically by alleviating the rate limiting steps which rely on cytochrome P-450. This in turn provides means of obtaining poppy plants with increased yield of alkaloids.

10 However, there will be instances where it may be preferable to manipulate the alkaloid metabolism of a poppy plant by suppression of genes encoding the P-450 reductases. The expression in the poppy of the cDNA encoding a P-450 reductase enzyme or part thereof, in an antisense orientation can be used to achieve this such that the expression directs the inhibition of the endogenous cytochrome P-450 reductase gene or homologues. In addition, the cDNA encoding the P-450 reductase enzyme or part thereof could be expressed in the sense orientation to direct the co-suppression of the
15 endogenous cytochrome P-450 reductase gene or homologues. Furthermore, the cloned cDNA sequence can be used to design ribozyme sequences such as the hammerhead or hairpin ribozymes that can be used to suppress the target gene by inactivation of the endogenous cytochrome P-450 reductase gene mRNA. The genes encoding the sense, antisense or ribozymes can be delivered as transgenes stably integrated into the poppy
20 genome or transiently in the form of a viral vector.

Although the invention has been described with reference to specific embodiments, modifications that are within the knowledge of those skilled in the art are also contemplated as being within the scope of the present invention.

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REFERENCES

1. Madyastha, K. M., Meehan, T. D., and Coscia, C. J. (1976) *Biochemistry* **15**, 1097-1102.
2. Rueffer, M., and Zenk, M. H. (1987) *Tetrahedron Lett.* **28**, 5307-5310.
3. Tanahashi, T., and Zenk, M. H. (1990) *Phytochemistry* **29**, 1113-1122.
- 5 4. Bauer, W., and Zenk, M. H. (1991) *Phytochemistry* **30**, 2953-2961.
5. Zenk, M. H., Gerardy, R., and Stadler, R. (1989) *J. Chem. Soc., Chem. Commun.* 1725-1727.
6. Stadler, R., and Zenk, M. H. (1993) *J. Biol. Chem.* **268**, 823-831.
7. Kraus, P. F. X., and Kutchan, T. M. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 2071-2075.
- 10 8. Madyastha, K. M., and Coscia, C. J. (1979) *J. Biol. Chem.* **254**, 2419-2427.
9. Fujita, M., and Asahi, T. (1985) *Plant Cell Physiol.* **26**, 397-405.
10. Benveniste, I., Gabriac, B., and Durst, F. (1986) *Biochem. J.* **235**, 365-373.
11. Kochs, G., and Grisebach, H. (1989) *Arch. Biochem. Biophys.* **273**, 543-553.
- 15 12. Hakamatsuka, T., Hashim, M., Ebizuka, Y., and Sankawa, U. (1991) *Tetrahedron* **47**, 5969-5978.
13. Menting, J. G. T., Cornish, E., and Scopes, R. K. (1994) *Plant Physiol.* **106**, 643-650.
14. Shet, M. S., Sathasivan, K., Arlotto, M. A., Mehdy, M. C. and Estabrook, R. W. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2890-2894.
- 20 15. Meijer, A. H., Lopes Cardoso, M. I., Voskuilen, J. T., de Waal, A., Verpoorte, R., and Hoge, J. H. C. (1993) *Plant J.* **4**, 47-60.
16. Pompon, D., Louerat, B., Bronine, A., and Urban, P. (1996) *Methods Enzymol.* **272**, 51-64.
- 25 17. Linsmaier, E. M., and Skoog, F. (1965) *Physiol. Plant* **18**, 100-127.
18. Gundlach, H. Müller, M. J., Kutchan, T. M., and Zenk, M. H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2389-2393.
19. Diesperger, H., Müller, C. R., and Sandermann, H. Jr. (1974) *FEBS Lett.* **43**, 155-158.
- 30 20. Shet, M. S., Sathasivan, K., Arlotto, M. A., Mehdy, M. C. and Estabrook, R. W. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2890-2894.
21. Eckerskorn, C., and Lottspeich, F. (1989) *Chromatographia* **28**, 92-94.
22. Cullin, C. and Pompon, D. (1988) *Gene* **65**, 203-217.

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